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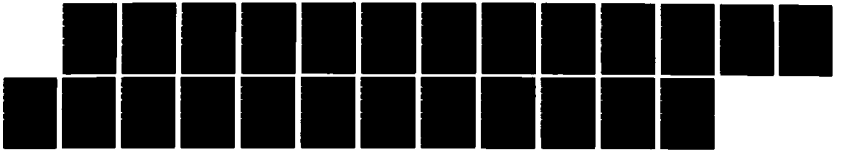

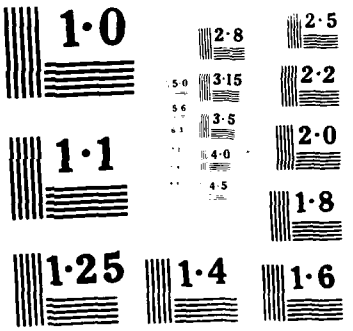
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Mechanisms of Rapid Nonspecific Resistence Induced by Immunomodulators: Delineation Using Selective Depletion of Cells

12 PERSONAL AUTHOR(S)

Page S. Morahan, Ph.d., Alvin Volkman, M.D.

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Macrophages, monocytes, PMN, NK cells, herpes simplex type 2, encephalomyocarditis virus, Listeria, host defense, immunotherapy, interferon, immunomodulators, C. Parvum, MVE-2, Muramyl dipeptide, lipoidal amines, 89Sr, bone marrow

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

A variety of immunomodulators have been evaluated in normal mice for ability to enhance nonspecific host resistance against herpes simplex virus type 2 (HSV-2), encephalomyocarditis virus (EMC), and Listeria monocytogenes. Several novel chemical immunomodulators were identified that had potent antiviral activity on prophylactic administration; these included CL246,738, avridine in liposomes, and several pyrimidinones. In addition, repeated therapeutic administration of either alpha or gamma interferon was effective, particularly against HSV-2 infection. The mechanism of action of these agents will be investigated in mice selectively depleted of circulating monocytes and of NK cells by 89Sr treatment. We have previously shown that several immunomodulators are effective in 89Sr treated mice, suggesting a major role for tissue macrophage activation for their effects. The present results have also shown that treatment regimes effective against the viral infections may not be effective, or may be adverse, against Listeria infection. The reasons are as yet undefined.

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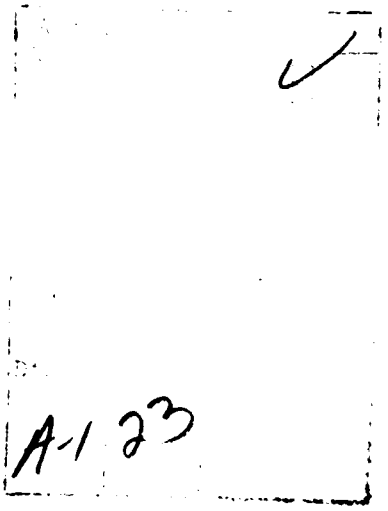
Work in the 89Sr model of selective cellular depletion has also continued to demonstrate the existence of a tissue macrophage population that is regulated independently of the bone marrow and circulating monocyte. This concept has been further strengthened by work with the Sl/Sld mouse, which is congenitally monocytopenic.

We have investigated changes in macrophage prostaglandin production produced by C. parvum treatment in normal, 89Sr treated, and Sl/Sld mice. Considerable support has been found for the definition of independently regulated mononuclear phagocyte populations in the spleen and peritoneal cavity.



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PROGRESS REPORT - 1 July 1985 - 30 June 1986

1.0 Detailed Progress Report

The report follows the specific objectives that were initially outlined for this program. Several manuscripts have been published during the year, and others submitted for publication. We have included the abstracts of these in the body of the text as the progress report for completed work; the reprints have been submitted previously as Technical Reports. We have only included detailed information for unpublished work.

1.1 Continue to evaluate the efficacy of immunomodulators in our battery of microbial infections.

We have completed our basic analysis of the effects of 89Sr treatment in the CD1 mouse on natural and immunomodulator induced host resistance. Our results establish that the antimicrobial activity of a diverse group of immunomodulators is independent of normal levels of circulating monocytes and NK cells. Rapid enhancement of nonspecific antimicrobial resistance may thus be achieved in profoundly immunosuppressed individuals, such as those undergoing chemotherapy for malignancy, receiving immunosuppressive drugs for organ transplants or autoimmune diseases, receiving radiation treatment or suffering radiation damage. The present data support the concept that the tissue macrophages (MΦ) in target organs for microbial pathogenesis may be sufficient for immunomodulator enhanced resistance.

We have also completed a study documenting the problems that inapparent infection of mice with murine hepatitis virus (MHV) may have on interpretation of experiments with MΦ. This study, performed in collaboration with Dr. Abigail Smith, Yale University, is the first to our knowledge that shows the kinetics of effects on MΦ after exogenous infection with MHV. Most previous data have resulted from retrospective studies, where the relationship could not be documented as precisely.

We have received a variety of potentially interesting immunomodulators for evaluation, and are evaluating them for ability to increase host resistance to encephalomyocarditis virus (EMC), herpes simplex virus type 2 (HSV-2) or *L. monocytogenes* infection. We are also establishing for some compounds their effects on MΦ, by measuring possible changes in surface ectoenzyme profiles and acquisition of antitumor activity. Some of the compounds and their sources are listed in Table 1. We have found only a few of the new immunomodulators to be as effective as our "standard" maleic anhydrde divinyl ether (MVE-2) or the killed vaccine of *C. parvum.* We are manipulating doses and schedules in an attempt to identify protective regimens.

Table 1. New Immunomodulators Currently Being Evaluated

Compound Name	General Structure	Source
	--Chemicals--	
LS 2616	quinoline-3-carboxamide	Dr. T. Stalhandske AB Leo Research Labs, Helsingborg, Sweden
ABPP (U-54,461)	2-amino-5-bromo-6-phenyl- 4(3H)-pyrimidinone	Dr. H. Renis Upjohn Company Kalamazoo, MI
AIPP (U-54,462)	2-amino-5-iodo-6-phenyl- 4(3H)-pyrimidinone	Dr. H. Renis
ABMP (U-25,166)	2-amino-5-bromo-6-methyl- 4(3H)-pyrimidinone	Dr. H. Renis
MA-CDA 1-10K,10-30K,>30K	cyclohexyl-1,3-dioxepin maleic anhydride copolymer	Dr. R. Ottenbrite Virginia Commonwealth University Richmond, VA
MA-MP (1-10K,10-30K,>30K)	4-methyl-2-pentenoyl maleic anhydride copolymer	Dr. R. Ottenbrite
IA-ST (1-10K,10-30K,>30K)	itaconic acid-styrene- copolymer	Dr. R. Ottenbrite
Immuthiol	sodium diethyldithiocarbamate	Dr. G. Renoux BioMerieux Tours, France
ADA 202-718	ethylene-2,2'-bis(dithio) bis(ethanol)	Dr. P. Hiestand Sandoz Ltd. Basel, Switzerland
CL 259,763	N-[4-[(4-fluorophenyl)sulfonyl] phenyl] acetamide	Dr. F. Durr Lederle Laboratories Pearl River, NY
CL 246,738	3,6-bis(2-piperidinoethyoxy) acridine trihydrochloride	Dr. F. Durr
	--Biologicals--	
rIFN-alpha	Human recumbinant alpha A/D interferon	Dr. P.F. Sorter Hoffman LaRoche Nutley, NJ
rIFN-gamma	Murine recumbinant gamma interferon	Dr. H.M. Shephard Genentech, South San Francisco, CA
IL-1	Recombinant mouse interleukin 1	Dr. P. Lomedico Hoffman-La Roche Nutley, NJ
	--Microbial Products--	
MPL	Monophosphoryl lipid A	Dr. E. Ribi Ribi ImmunoChem Hamilton, MT
TDM	Trehalose dimycolate	Dr. Ribi
MTP-PE	Muramyl tripeptide- phosphatidylethanolamine	Dr. P. Dukor Ciba-Geigy Basel, Switzerland

MDP	Muramyl dipeptide	Dr. L. Chedid Institute Pasteur Paris, France
MDP-GDP	Lipophylic MDP	Dr. Chedid
EP-LPS	B. pertussis endotoxin associated protein	Dr. A. Winters University of Alabama University, AL

Abstracts of our published manuscripts for this specific aim include:

Abstract: Morahan, P.S., E.R. Leake, D.J. Tenney, and M. Sit. Comparative analysis of modulators of nonspecific resistance against microbial infections. In: J. Majde, Ed. Immunological Adjuvants and Modulators of Non Specific Resistance to Microbial Infections (Alan R. Liss, NY).

Three immunomodulators, pyran, MVE-2 and C . parvum , provided significant protection with prophylactic administration against the three infections tested. Several synthetic immunomodulators were very effective with prophylactic administration against both EMC and HSV-2 viral infections; these included CL246,738, the pyrimidinones, and avridine in liposomes. Recombinant alpha and gama IFNs and natural beta IFN were effective on repeated therapeutic treatment against both EMC and HSV-2; alpha and gamma IFN were signficantly more effective than beta IFN, and were more effective against HSV-2 than EMC. The efficacy of IFNs against Listera needs to be evaluated. Certain immunomodulator regimens were protective against viral infections but enhanced Listeria infection; this is t oubling for clinical potential. It points out the need for more systematic comparative investigation of immunotherapy in a variety of infection models. At the same time, the fact that a given drug regimen can inhibit or enhance an infectious disease process provides an experimental tool to better define pathogenesis and resistance mechanisms important in different microbial processes.

Abstract: Morahan, P.S., W.L. Dempsey, A. Volkman and J. Connor. Antimicrobial Activity of Various Immunomodulators: Independence from Normal Levels of Circulating Monocytes and NK Cells. Infec. Immun. 51:87-93, 1986.

The effects of 89Sr treatment on natural host resistance of CD1 mice, and on enhancement of resistance by immunomodulators, to infection with Listeria monocytogenes or herpes simplex virus type 2 (HSV-2) were determined. In the CD1 mouse, single dose treatment with 89Sr caused, within one week, a profound decrease in the number of circulating monocytes (Mo), lymphocytes (Ly), and polymorphonuclear leukocytes (PMN). There was also marked functional impairment of the Mo inflammatory response, as well as markedly decreased spontaneous and activatable cytotoxicity by splenic natural killer (NK) cells. Despite this profound cellular suppression, there was no significant change in natural resistance of CD1 mice to L. monocytogenes or HSV-2 infection. Furthermore, prophylactic treatment of mice with the biologic immunomodulator, C. parvum, or the synthetic immunomodulators, MVE-2 or avridine in liposomes, resulted in comparable enhancement of resistance in 89Sr treated mice and in normal mice.

These data indicate that natural and immunomodulator-enhanced resistance of CD1 mice to microbial infections do not depend upon normal levels of Mo, PMN,

or NK cells. The resistance enhancement may rely on activated tissue macrophages (MO). In contrast to the early changes in circulating WBC, the resident peritoneal cell populations were not markedly altered until after 30 days. There then was a distinct decline in Ly and a gradual decline in MO; the change in MO was apparently due to lack of an age-related increase in the peritoneal MO population in 89Sr treated mice in comparison with a slight increase in resident MO in normal mice. After 89Sr treatment of CD1 mice, the number of PMN and the function of NK cells generally recovered by about day 50, and was followed by partial recovery of circulating MO, unless a second dose of 89Sr was administered.

Abstract: Dempsey, W.L., A.L. Smith and P.S. Morahan. Effect of Inapparent Murine Hepatitis Virus Infections on Macrophages and Host Resistance. J. Leuk. Biol. 39:559-566, 1986.

Inapparent infections of mice with murine hepatitis virus (MHV) altered host resistance to experimental infection with a second virus, encephalomyocarditis virus (EMC), reduced the protective effects of exogenously administered interferon against EMC infections, and altered macrophage ectoenzyme phenotypes in two macrophage populations. Resident peritoneal macrophages from mice experimentally infected with one of two strains of MHV also demonstrated altered ectoenzyme phenotypes. These data demonstrate that inapparent infections with MHV alter several host resistance and macrophage parameters, and directly demonstrate that effects of inapparent MHV infection on macrophage parameters can be reproduced experimentally.

1.2 Define the effects of immunomodulators on various nonspecific effector cell populations in the 89Sr system.

Dr. Volkman and Dr. Shibata have continued their investigations into the immunomodulatory effects of C. parvum in mice deficient in circulating monocytes. Two models of monocyte deficient mice are being employed. One involves the destruction of monocyte precursors in the bone marrow with the bone-seeking isotope strontium-89 (89Sr) which has a T1/2 of about 50 days (β ,max.1.4 MeV). This system was discussed in detail in earlier progress reports, and essentially involves the iv administration of the isotope at a dose of 2-4 Ci/gram body weight. Approximately 50% of the dose is immediately incorporated into bone matrix; the rest in rapidly excreted. Such treatment results in the rapid destruction of bone marrow, profound monocytopenia, and a marked increase in splenic hemopoiesis which does not offset the monocytopenia. The peritoneal MO population, by contrast, remains stable for at least 30 days.

The second model employs the congenitally monocytopenic S1/S1d mouse, which we have shown to be profoundly monocytopenic. Monocyte-MO elicitation is poorly demonstrable in these mice but peritoneal MO appear to be unaffected. In essence, the S1/S1d mouse supports the observations of the 89Sr model while escaping the criticism that the apparent stability of the peritoneal MO population is due to a long turnover time.

The focus in this project period has been largely on arachiodnic acid (AA) metabolism and release of eicosanoids by splenic and peritoneal macrophages. By way of rationale, differences in AA metabolism in different populations of MO is well known and may possibly provide a clue that would link MO subpopulations. The prostaglandin most frequently assessed has been PGE2, principally by radioimmunoassay. This fatty acid appears to be the agent largely responsible for the suppressive effect of C. parvum modulated splenic macrophages on T cell proliferation, and also provides an assessment of the integrity of the cyclooxygnase pathway. The results have shown that the spontaneous release of PGE2, induced by C. parvum administered intraperitoneally, is abrogated by prior treatment of mice with 89Sr. This effect is not attributable to the monocytopenia because the response of the profoundly monocytopenic S1/S1d mice to C. parvum is normal or increased. In brief, failure of the capacity of C. parvum to induce PGE2 producing splenic macrophages appears to be a reflection of a bone marrow dependent function.

Results have also shown that splenic macrophages from bone marrow impaired mice do not release PGE2 upon stimulation in vitro with zymosan, phorbol ester (PMA) or calcium ionophore. These findings suggest that the altered response to C. parvum treatment in vivo is not due to the activities of an inhibitory mechanism, but to the intrinsic metabolic state of the splenic macrophages following 89Sr treatment. The mechanisms underlying this altered AA cascade are uncertain but does not involve any difference in the incorporation of arachidonic acid (AA) as shown by quantitatively normal uptake of tritiated AA (3H-AA). The distribution of 3H-AA among membrane phosphatides was also comparable in 88Sr (control) and strontium-89 treated mice. In sharp contrast, peritoneal macrophages, irrespective of whether they had been collected from test or control mice, released significantly large quantities of PGE2 on in vitro stimulation with zymosan, PMA or calcium ionophore.

A recent extension of the eicosanoid investigations has been done with the generous collaboration of Dr. John Humes of the Merck Research Institute, Rahway, New Jersey. This aspect of the project is concerned with the evaluation of another metabolic pathway of AA, the 5-lipoxygenase pathway, leading to the formation of leukotriennes C4 (LTC4) and B4 (LTB4). An unexpected finding was that LTC4 was not produced in detectable amounts by splenic macrophages before or after 89Sr treatment. Neither C. parvum nor calcium ionophore have been effective in reversing this result. It should be noted that the assay is said to be sensitive to a lower limit of approximately

0.5 nanograms. Peritoneal macrophages, by contrast, release significantly large quantities of LTC4 irrespective of whether they have been collected from test or control mice. Evaluation of LTB4 is currently in progress. Failure to detect LTB4 would suggest absence of the parent compound (LTA4) which could reflect inactivity of the enzyme 5-lipoxygenase. There are, however, possble flaws in this interpretation. One is that lipoxygenase poducts are beng formed at levels below the sensitivity of the assay. Another is that other products, not assayed, are being formed preferentially. Additional studies are being conducted to clarify these problems.

Abstracts of published and submitted manuscripts follow below.

Abstract: Selectively Eliminated Blood Monocytes and Splenic Suppressor Macrophages in Mice Depleted or Bone Marrow by Strontium 89, Yoshimi Shibata, Walla L. Dempsey, Page S. Morahan, and Alvin Volkman, Journal of Leukocyte Biology 38:659-669, 1985.

The contribution of specific activity to the effects of the bone-seeking isotope, strontium 89 on radiosensitive components of mononuclear phagocyte populations was investigated in mice. CBA/J mice received a fixed dose of 2 uCi/g body weight of 89Sr with three different radioactivity located in the bone surface was 4,200, 3,000, and 2,400 cpm/mg bone when measured 2 days after the administration of 89Sr, and was lost with an estimated biological half-life of 27, 25, and 23 days, respectively. Bone marrow suppression was assessed by quantitation of the depletion of macrophage-colony forming cells (M-CFC) grown in vitro in the presence of macrophage growth factor. The decline in M-CFC closely paralleled the level of radioactivity in the bone. These effects were clearly reflected by the depletion of monocytes in the blood, which were reduced to 14%, 14%, and 21% of control levels corresponding to SA's of 6 uCi/mg, 100 uCi/mg and 20 uCi/mg when counted on day 10. By day 30 the respective monocyte level were 15%, 31% and 77%. Furthermore, the induction of prostaglandin E producing suppressor macrophages (MO) by Corynebacterium parvum administration was found to vary inversely with the effects of radioactivity in the bone, with initial impairment followed by quantitative recovery. Resident-type MO in peritonel cavity, however, appear to be unaffected by 89Sr-treatment. These data suggest, as before, that the monocytes and suppressor MO are dependent on raiosensitive marrow cells. The observations also lead to the conclusion that the specific activty of 89Sr preparations is an important determinant of the degree of suppression and the rate of recovery of bone marrow from the effects of irradiation that follow the administration of this isotope.

Abstract: The effects of Bone Marrow Depletion of Prostaglandin E-Producing Suppressor Macrophages in Mouse Spleen, Yoshimi Shibata and Alvin Volkman, Journal of Immunology, 13 5:6, 3897,3904, 1985.

The i.p. injection of Corynebacterium parvum (CP) into CBA/J mice effected increases in macrophage colony-forming cells (M-CFC) when spleen cells were cultured with L cell culture filtrate as a source of colony-stimulating factor. Significant increases in phagocytic macrophages (MO) with Fc receptors for IgG2a and IgG2b immune complexes were additionally noted among the spleen cells in these mice. These MO effectively inhibited Con A-induced lymphocyte proliferation, probably reflecting a 10-fold increase above normal controls in prostaglandin E to 47 ng/3 x 10^6 spleen cells/ml. To determine whether the suppressor MO are immediate derivatives of splenic M-CFC, we tried to induce suppressor MO by the injection of CP into mice depleted of bone marrow M-CFC by the earlier administration of the bone-seeking isotope, 89Sr. This procedure reduced M-CFC in the bone marrow to less than 1% of normal for more than 30 days. Monocytes in the blood fell to 5% of normal by day 10 and were 30% on day 30. Levels of resident peritoneal MO showed relatively little change in this period. By contast, splenic M-CFC increased to 20-fold higher than the "cold" 88 Sr controls. CP-induced suppressor MO activity, however, was sharply reduced in 89Sr marrow-depleted mice on day 10, despite the striking increase in M-CFC. There was a threefold increase in the number of phagocytic MO binding IgG2a immune complexes, with no significant increase in IgG2b binding MO. The kinetics of recovery of suppressor MO activity showed that on days 20, 30, and 50 after 89Sr injection the activities reached 20%, 30% and 70% of the "cold" control, respectively, and correlated with the recovery of significant levels of M-CFC in the bone marrow. Taken together, these observations suggest that splenic M-CFC are not an immediate source of PGE-suppression MO in vivo. It appears more likely that the CP-induced suppressor MO, in particular, originate from radiosensitive bone marrow cells or require for differentiation a microenvironment provided by bone marrow cells. The data also suggest that the expression of the FcY2b receptor and or suppressor activity by CP-induced splenic MO are related phenomena.

Abstract : The Effect of Hemopoietic Microenvironment on Splenic Suppressor Macrophages in Congenitally Anemic Mice of Genotype S1/S1d, Yoshima Shibata and Alvin Volkman, Journal of Immunology, 135:6, 3905-3910, 1985.

Mechanisms underlying mononuclear phagocyte specialization are being probed by studying suppressor macrophages (MO) as a reference population in mouse models with impaired blood monocyte formation. Splenic suppressor MO, defined by PGE-mediated inhibiton of Con A-induced T lymphocyte proliferation are induced by the i.p. administration of Corynebacterium parvum (CP). Mice severely depleted of bone marrow and blood monocytes by treatment with 89Sr fail to show this suppressor MO response to CP, although MO-forming stem cells, assessed as splenic M-CFC in vitro, are increased 20-fold. These observations suggest that radiosensitive bone marrow stem cells are necessary for the generation of both suppressor MO and monocytes and that one such stem cell may be common to both types of mononuclear phagocytes. This notion was explored further by employing congenitally anemic mice of the genotype S1/S1d in which the hemopoietic microenvironment is genetically defective and thus unable to support the proliferation, differentiation, and function of stem cells. The congenital defect was found to be additionally expressed in the S1/S1d mouse by a monocytopenia of less than 10% of the values in normal congenic litermate controls and by the failure of splenic M-CFC to increase in response to CP. PGE-producing suppressor MO expressing FcY2b receptors, however, were induced by CP in S1/S1d mice with no significant diminution of suppressor activity. These data establish he fact that significant impairment of the formation of monocytes

is part of the overall hemopoietic defect in S1/S1d mice. PGE-producing suppressor MO, however, were inducible at normal functional levels in the presence of a profound monocytopenia, and therefore appear to be independent of the mechanisms that regulate blood monocyte formation. Ablation of the bone marrow with 89Sr resulted in failure of CP to induce suppressor MO in the spleens of the S1/S1d mice as in the littermate controls. Other observations in the present study, when taken with data from the 89Sr model, show the additional independence of these suppressor MO from splenic M-CFC. In aggregate, these findings delineate three functionally definable populations of mononuclear phagocytes that appear to be independently regulated.

Abstract : Definition of Independently Related Mononuclear Phagocyte Populations by Use of Eicosanoid Probes. Yoshima Shibata, Abraham P. Bautista, Sam N. Pennington, John L. Humes and Alvin Volkman, American Journal of Pathology, submitted for publication.

Bone marrow depletion effected with the bone seeking isotope, 89Sr, was employed in CBA/J mice as a model in which to facilitate the definition of mononuclear phagocyte populations. Blood monocytes also become rapidly depleted in mice thus treated. Macrophages (MO's) isolated from the unstimulated peritoneal cavity and the spleen following ip administration of Corynebacterium parvum (CP) were assessed for their capacity to suppress Con A stimulated T-lymphocyte proliferation in conjunction with the synthesis and release of prostaglandin E2 (PGE2) and leukotriene C4 (LTC4).

Concentrations of PGE2 and LTC4 in the culture medium were measured by radioimmunoassay; 3H-PGE2 release following the uptake of 3H-arachiodonic acid by membrane phospholipids was determined by thin-layer chromatography. PGE2 production by splenic suppressor MO's in intact mice increased tenfold by 7 days after ip CP (62 mg/3 x 10^6) cells/ml). Neither the suppressor nor the PGE responses, however, could be included in the corresponding MO's from bone marrow depleted mice despite an increase in the number of splenic MO's. In vitro treatment of the latter MO's with either Con A, zymosan, calcium ionophore A23187 or phorbol ester likewise failed to stimulate the release of PGE2. A striking additional finding was that no release of LTC4 was detectable in splenic MO's from either intact or bone marrow depleted mice. Unlike the behavior of splenic MO's, the release of PGE2 and LTC4 by resident peritoneal MO's was unaffected by the depletion of bone marrow and blood monocytes. Increased release of PGE2 was demonstrable following the in vitro stimulation of resident peritoneal MO's with zymosan and A23187.

Taken together, the data define two distinct PGE2 producing MO populations with respect to a requirement for bone marrow. Thus, PGE2 production by resident peritoneal MO's is apparently independent of the status of the bone marrow, whereas this function in splenic suppressor MO's is demonstrably dependent on the presence of bone marrow. The data suggest, in addition, a distinct difference between splenic and resident peritoneal MO's in the expression of lipoxygenase pathway metabolism represented by the production of LTC4.

1.3 Develop effective methods to decrease tissue MO in normal and 89Sr treated mice.

The general thrust of this work is to prepare liposomes with incorporated cell toxins, in the form of particles to be phagocytized by macrophages. The eventual goal is to destroy local populations of macrophages

selectively while sparing nonphagocytic cells. Dr. Jendrasiak and Dr. Volkman have been engaged in attempts to optimize the preparation of liposomes for this purpose and to evaluate ricin A chain as a cytotoxin with the later goal of evaluating other toxins and -emitting isotopes such as astatine 211 for their efficacy in destroying local populations of tissue macrophages.

One phase of this work involves ongoing attempts to determine the optimum physical conditions of the liposome with respect to its uptake by mononuclear phagocytes. The model system for these and other exploratory studies in the macrophage-like cell line, P388D1, which provides an economical and rapid means of generating large numbers of cells for in vitro assays. We recognize a potential pitfall in the use of P388D1 cells, since the phagocytic and other properties of this cell line may be different from those of either resident or exudate type mouse macrophages. We therefore regard assays employing P388D1 cells as developmental tools.

In one series of experiments multilumellar liposomes of varying composition were prepared and incubated with cells, either in suspension or adherent to plastic dishes, at 37C and at 4C. In each instance, 3H cholesterol in trace quantities (10 Ci in about 0.03 ug/5ml liposomes) was added in preparation. In one experiment additional cholesterol was added to the phosphatide mixture to yield a molar ratio of 7:1:3 with respect to phosphatidylcholine (PC), phosphatidic acid (PA) and cholesterol. Five incubation intervals ranging from 5 min to 3hr were arbitrarily established.

Text figures 1-5 show that at 37C the constitution of liposomes in these experiments did not appreciably influence the level of uptake measured after 3 hr of incubation. Cells in suspension appeared to be a little more effective in liposomal uptake than adherent cells in monolayers, particularly at the 5-30 min intervals. The presence of significant levels of cholesterol (CH), that is, far greater than trace levels, appeared to result in a somewhat more rapid increase in cell associated radioactivity.

An interesting observation is that the data for uptake of liposomes at 37C and at 4C in monolayers strongly suggest that the observed differences are due to endocytosis at 37C and only weak binding to cell membranes at 4C.

Experiments are being conducted to determine optimal dose and time for attaining maximal toxicity following the administration of ricin A chain (RAC) bearing liposomes. In a first experiment, amino acid analysis, performed through the courtesy of Dr. Paul Fletcher, Shared Research Resources Unit, East Carolina University School of Medicine, was evaluated as a means of quantification of RAC incorporation into liposomes. An initial pilot preparation as performed in which 14 ug of RAC was added in 5 ml of aqueous phase to dried lipids. A second preparation with liposomes without RAC was also prepared. Triplicate suspension cultures of P388D1 cells in PBS were incubated with either RAC containing or RAC free liposomes for 2hr. After appropriate washing the viability of test and control cultures as assessed by their capacity to incorporate 3H leucine. The results (Fig.6) showed about a 40% reduction in leucine uptake by the cells incubated with RAC-liposomes. The amino acid analysis showed that all of the RAC had been incorporated into the liposomes. Determination of the limits of RAC incorporation based on amino acid analysis could not be pursued because of equipment failure. The amino acid analyzer, however, has recently been repaired and studies of this sort will be resumed.

Additional studies of RAC incorporation into liposomes have been carried out employing RAC that has been iodinated with 125I by Dr. Abraham Bautista in

Dr.Volkman's laboratory. In contrast to amino acid analysis, this procedure has indicated that the efficiency of RAC incorporation into liposomal vesicles, in concentrations ranging from 10 to 100 ug/ml, is only about 60%. The reason for this discrepancy is not clear and further studies addressing this problem are planned.

A dose response experiment was mounted employing P388D1 cells in suspension incubated in the presence of liposomes and ricin A chain (RAC) varying in concentration from 11.4 to 182 ug/ml of initial liposome preparation. Approximately 0.2 ml of liposomes was added to 1 ml of cell suspension containing 2 x 10^6 macrophages. Non-toxic liposomes and medium alone served as controls. After 6 hr of incubation at 37C the cells were washed and resuspended in fresh medium containing 3H leucine. A parallel experiment was carried out in which 50 mM galactose was incorporated into the medium to block the binding and uptake of any residual whole ricin or B chain residues that might have been present as a consequence of incomplete purification of RAC. The results are shown in Figures 7 and 8, each of which shows a dose-dependent decrease in leucine uptake. Despite the appealing geometry of the graphs, these data show that only a 14% reduction in leucine uptake was measured at the highest concentration of RAC. In the galactose medium the percent decrease in leucine uptake was 24%. This difference proved to be only marginally significant when examined statistically, but the fact that the blocking of galactose receptors did not result in increased survival indicated that no significant quantity of whole ricin had been present.

In an additional study, the question of survival of P388D1 cells under the suboptimal conditions conventionally employed for such assays was explored. It was considered particularly important to evaluate survival in phosphate buffered saline. Again, employing 3H-leucine uptake as a parameter of survival, it was found that in PBS survival by 3hr was already substantially below that in RPMI (see Figure 9). This experiment suggests a caveat concerning the interpretation of putative toxic effects attributed to ricin. Although appropriate controls would establish baselines for evaluating toxicity, the population under study may already be sufficiently impaired to reduce the sensitivity of leucine uptake as a measurement of viability.

Differences in lipsome uptake could strongly influence the cytotoxic effects of liposome-associated toxins. An experiment was therefore performed to compare liposome uptake by 3 putative target cell populations: Resident peritoneal MO, exudate (thioglycollate elicited) MO and P388D1 MO-like cells. Since the size of liposomes may also influence their uptake by phagocytic cells, liposome suspensions were passed through 0.2 and 0.6 μ nucleopore membrane ultrafilters before incubation with the respective cell populations. Cells were incubated in RPMI 1640 for 6 hr at 37° with and without variously filtered preparations of liposomes labeled with 3H-cholesterol. Liposome formulation was PC/PA/cholesterol prepared by ultrasonification as previously described. The results are summarized in Fig. 10 and show that liposomal uptake (cell-associated radioactivity) is highest in exudate MO and P388D1 cells and significantly lower ($p < .01$) in resident peritoneal MO. Liposome filtration appears to have been without effect possibly because of a high degree of uniformity in the preparation employed.

An additional comparison was made among putative target cells with respect to their capacity for leucine uptake. Cell types were the same as in the preceding experiment; incubation with 3H-leucine was carried out in otherwise leucine-free medium for 1 hr at 37 C except for cells previously incubated with liposomes for 3 hr. The results are summarized in Fig. 11 and show a dramatic difference in the capacity of the classes of test cells to incorporate leucine. This is undoubtedly a reflection of their respective levels of protein synthesis. It is important to note that resident peritoneal MO show so little uptake of leucine that this method is probably not sensitive enough to be used to quantify cytotoxicity. More appropriate methods will be sought.

Legends for Text Figures

Figures 1-5: The data in these text figures represent an experiment in which P388D1 cells were incubated for intervals of 5, 15, 30, 60 and 180 min either in suspension in phosphate buffered saline (calcium and magnesium free) or after having been plated on 35 mm plastic dishes and preincubated overnight to permit the formation of adherent monolayers. The initial number of P388D1 cells in each case was 2 x 106; liposomes were prepared of differing components indicated as PC (phosphatidylcholine), PA (phosphatidic acid), and CH (cholesterol). A radioactive label was introduced in the form of 3H-cholesterol estimated to contain about 30 picograms of carrier per 5 ml of liposome preparation. Calculations showed that only about 10% of the tritiated cholesterol was incorporated into the liposomes. This minute quantity of cholesterol is considered to be insignificant with respect to alteration of the character of the liposomes. Comparisons were made of the percent of available radioactivity accumulated by cells when incubated at 37 C or 4 C. Sampling intervals are shown along the X-axis as time in minutes to the log base 2 to compensate in part for the regularly spaced intervals. Overall the patterns for suspension cultures and those for cells in monolayers closely resemble each other irrespective of the constitution of the liposomes. Time and temperature appear to have a greater effect than liposomal constitution. As a matter of clarification, the total available radioactivity per sample was estimated to be approximately 0.2 uCi. This figure and the activity of the samples were determined by liquid scintillation counting.

Figure 6: The effect of adding increasing concentrations of ricin A chain (RAC)to dried PC, PA and CH (7:1:3) in the preparation of liposomes. The zero point represents the uptake of 3H-leucine by P388D1 cells incubated at 37 C with "empty" liposomes and is taken as 100%. Incubation time, 3 hr. RAC incorporation into liposomes was calculated to be 100% by amino acid analysis. Means of 4 determinations are shown.

Figures 7 and 8: The effect of the in vitro incubation (3 hr) with RAC-liposomes on 3H-leucine uptake by suspensions of P388D1 cells in the presence (Fig. 8) and absence (Fig. 7) of 50 mM D-galactose. The bars represent the means of 4 preparations.

Figure 9: Uptake of 3H-leucine as an index of viability of P388D1 cells cultured in suspension at 37 C, 7.5% ofCO2 and full humidity. Results compared RPMI 1640 plus 10% FBS with PBS (Ca^{++} and Mg^{+} free). 3H-leucine was added to cultures sampled at 3, 6, 12 and 24 hr of incubation (expressed as log base 2).

Figure 10: Uptake of unfiltered and ultrafiltered liposomes by resident peritoneal MØ (REM), exudate (thioglycollate elicited) MØ (EXM) and P388D1 cells. Decimals represent filter pore size in μ. See text for details.

Figure 11: Uptake of 3H-leucine by resident peritoneal MØ (REM), exudate (thioglycollate elicited) MØ and P388D1 cells. Preincubation with liposomes is designated by LIP. See text for details.

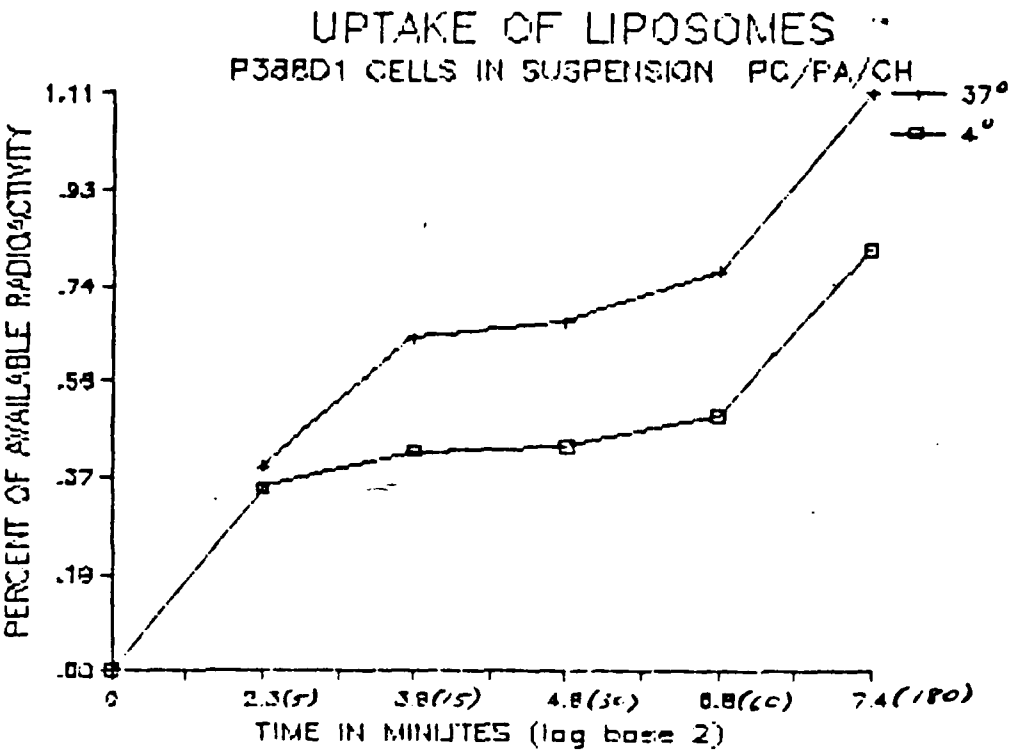

Fig 1.

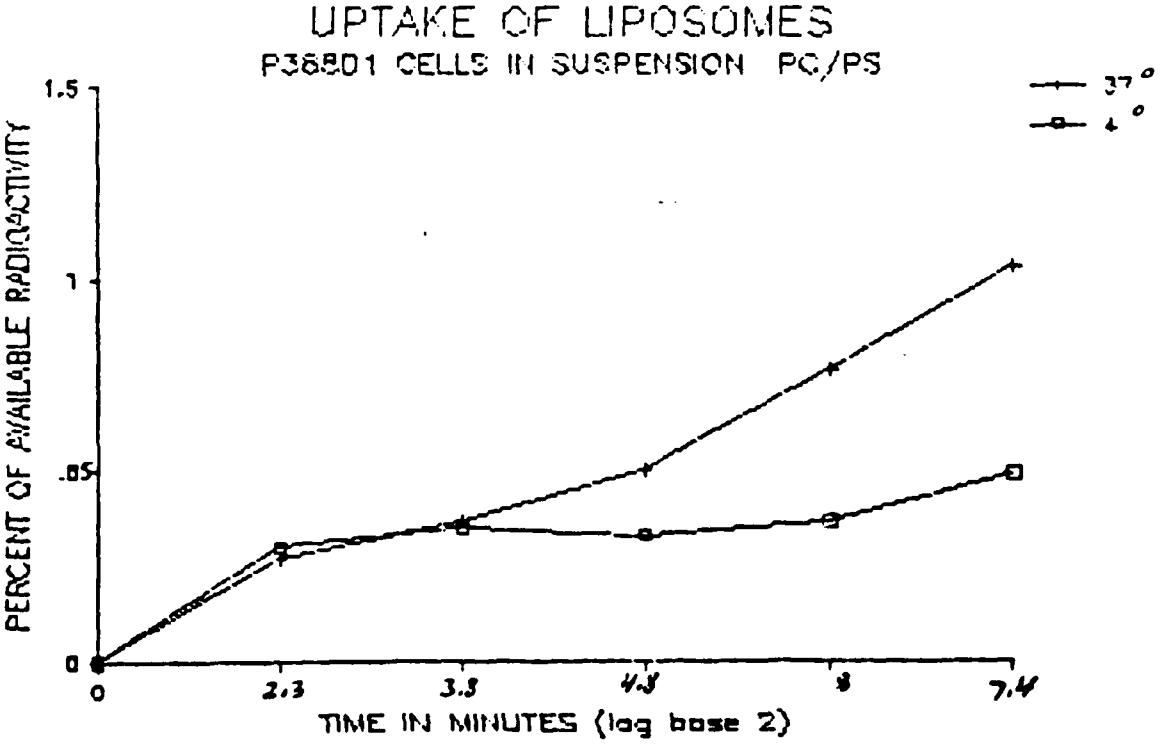

Fig 2.

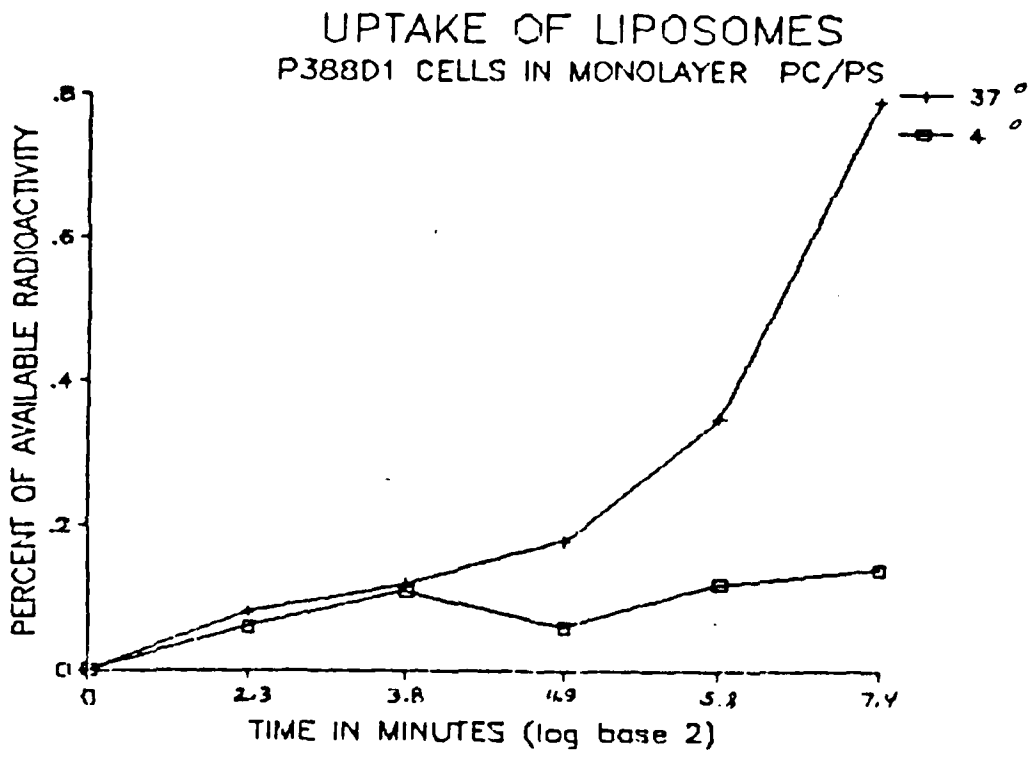
UPTAKE OF LIPOSOMES
P388D1 CELLS IN MONOLAYER PC/PS
37°
4°
PERCENT OF AVAILABLE RADIOACTIVITY
.8
.6
.4
.2
0
0
2.3
3.8
4.9
5.8
7.4
TIME IN MINUTES (log base 2)

Fig 3.

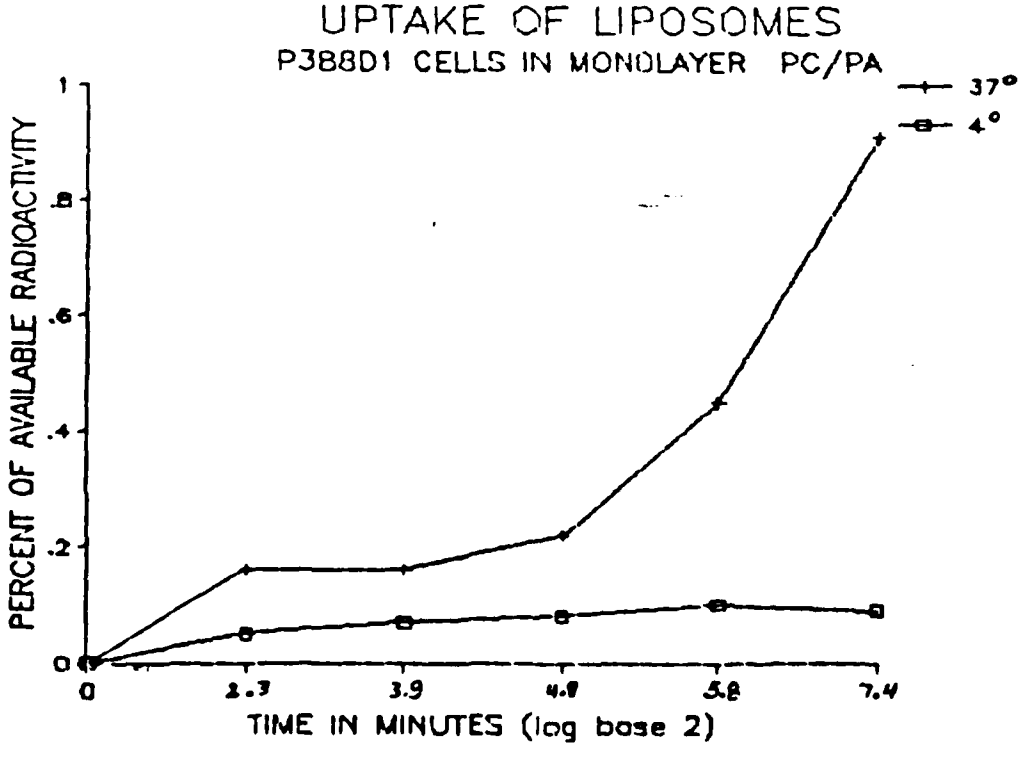
UPTAKE OF LIPOSOMES
P388D1 CELLS IN MONOLAYER PC/PA
37°
4°
PERCENT OF AVAILABLE RADIOACTIVITY
1
.8
.6
.4
.2
0
0
2.3
3.9
4.8
5.8
7.4
TIME IN MINUTES (log base 2)

Fig. 4

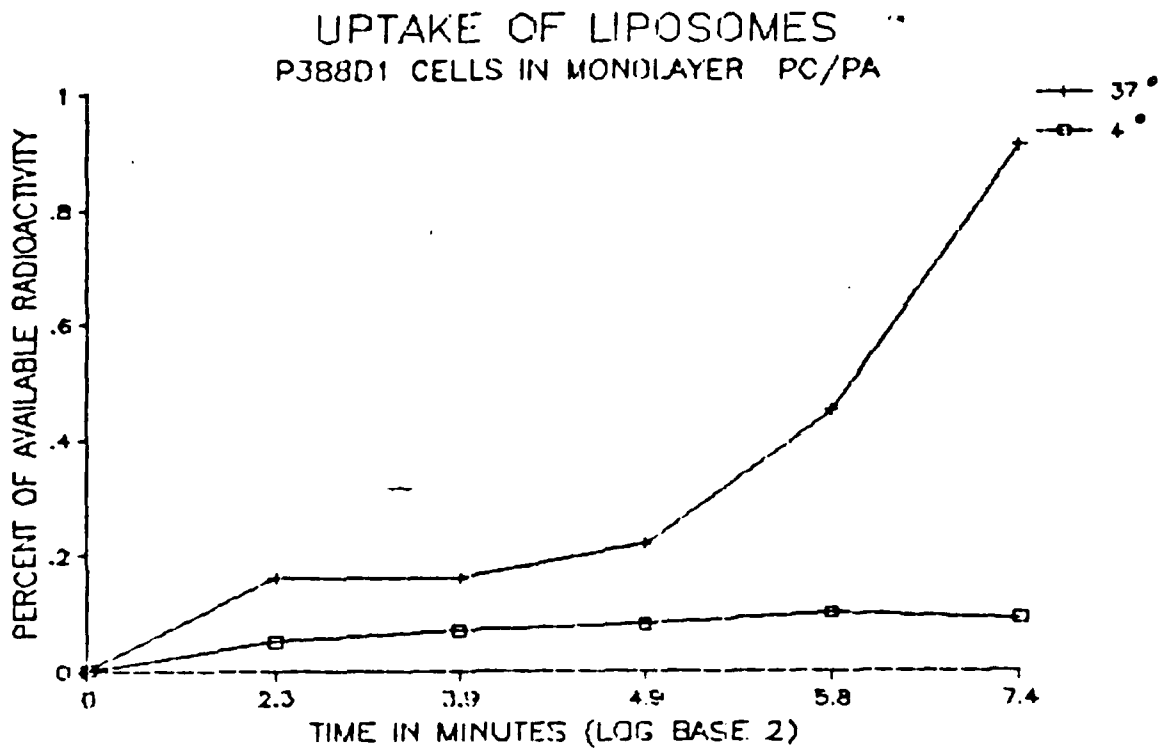

Fig 5

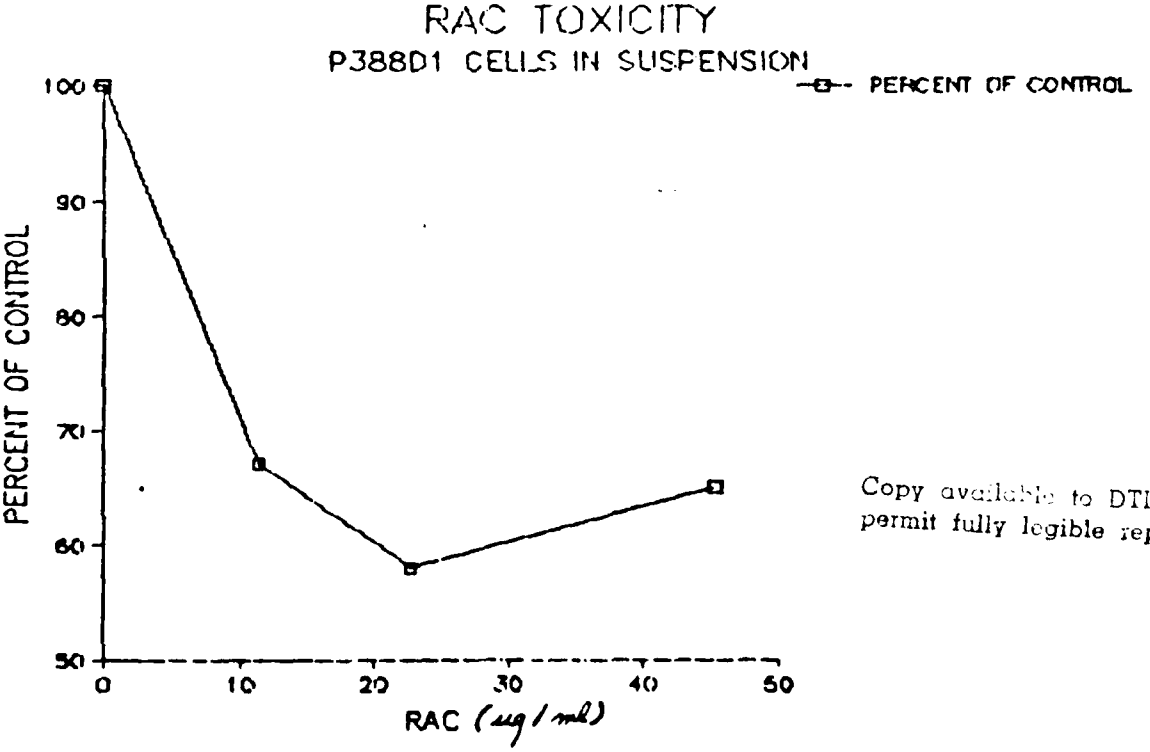

Fig. 6

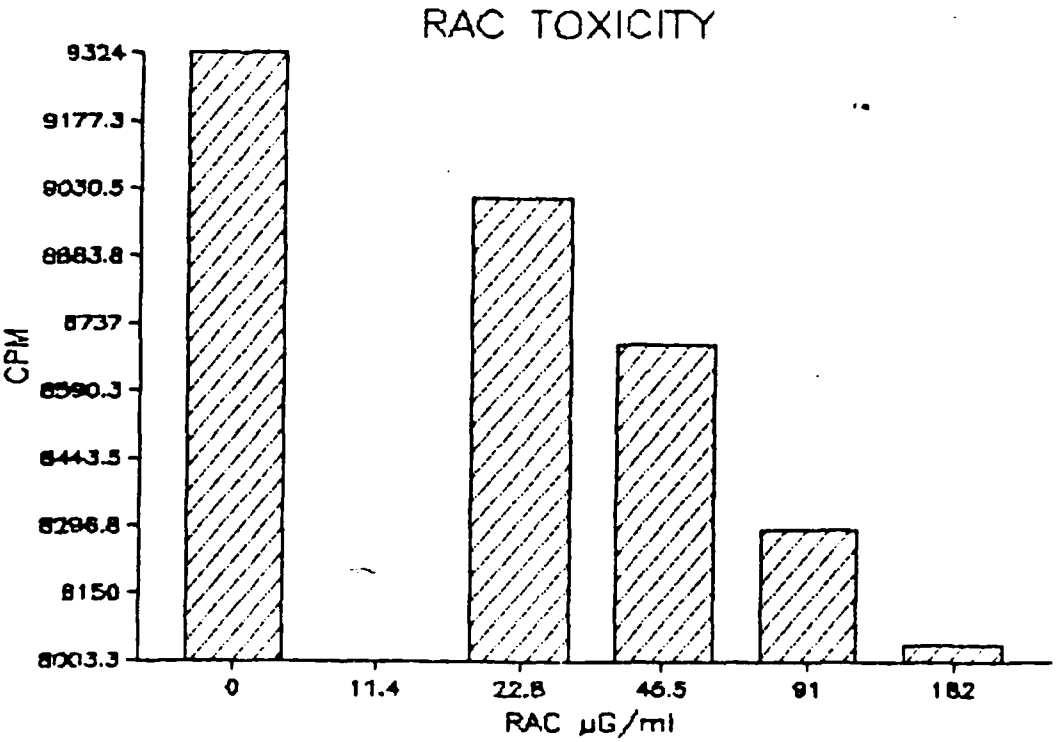
RAC TOXICITY
9324
9177.3
9030.5
8883.8
8737
8590.3
8443.5
8296.8
8150
8003.3
CPM
0
11.4
22.8
46.5
91
182
RAC µG/ml

Fig 7.

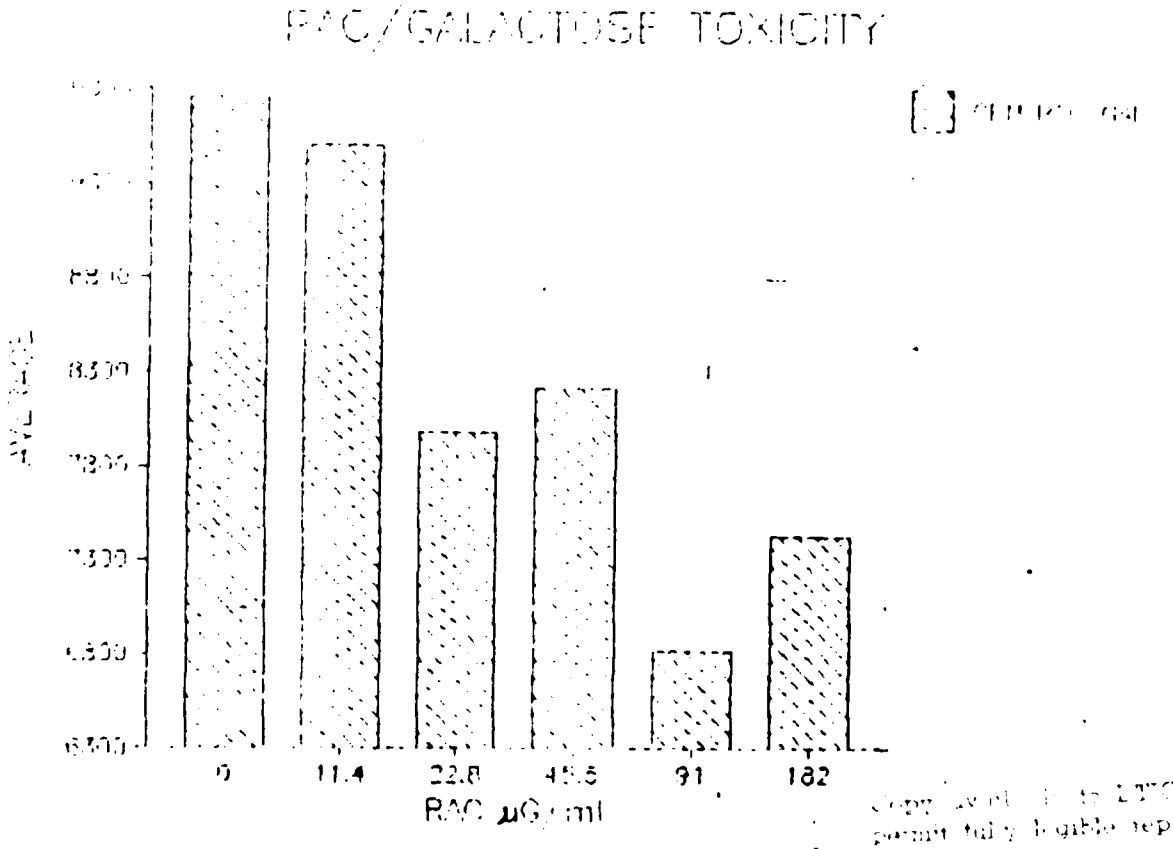
RAC/GALACTOSE TOXICITY
0
11.4
22.8
46.5
91
182
RAC µG/ml

Fig 8.

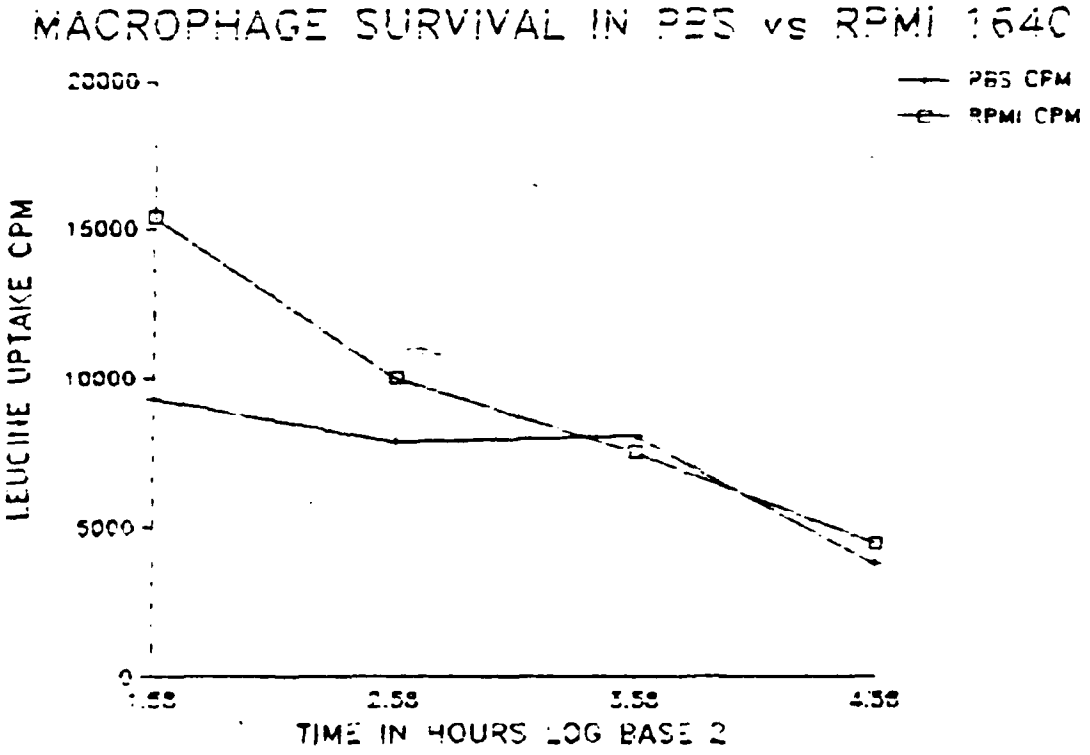

Fig 9.

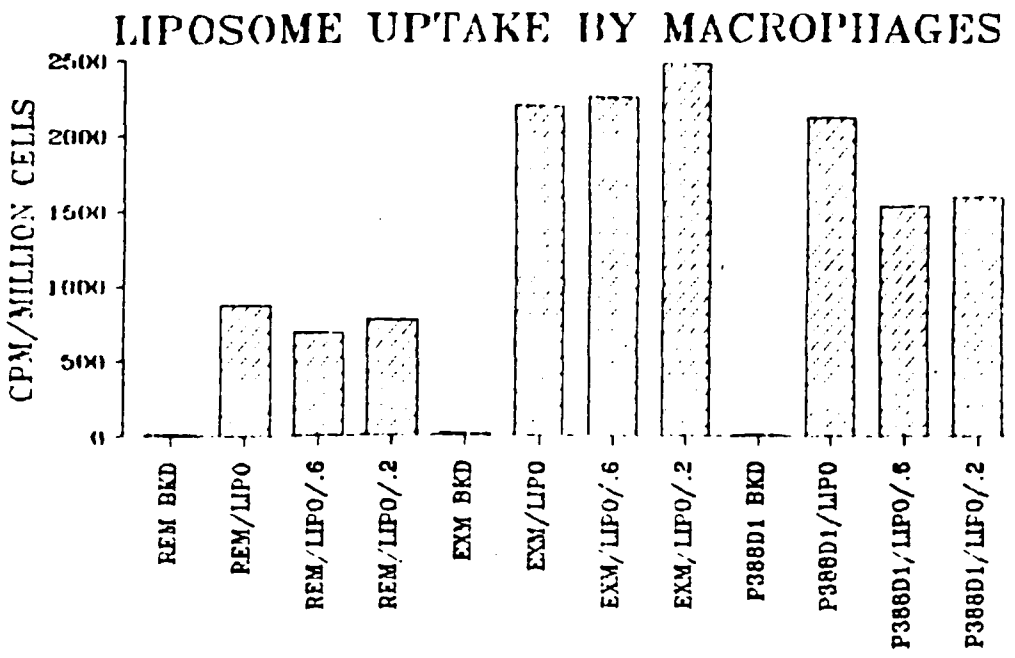
LIPOSOME UPTAKE BY MACROPHAGES
CPM/MILLION CELLS
2500
2000
1500
1000
500
0
REM BKD
REM/LIPO
REM/LIPO/.6
REM/LIPO/.2
EXM BKD
EXM/LIPO
EXM/LIPO/.6
EXM/LIPO/.2
P388D1 BKD
P388D1/LIPO
P388D1/LIPO/.6
P388D1/LIPO/.2
TYPE OF MACROPHAGE

Fig. 10.

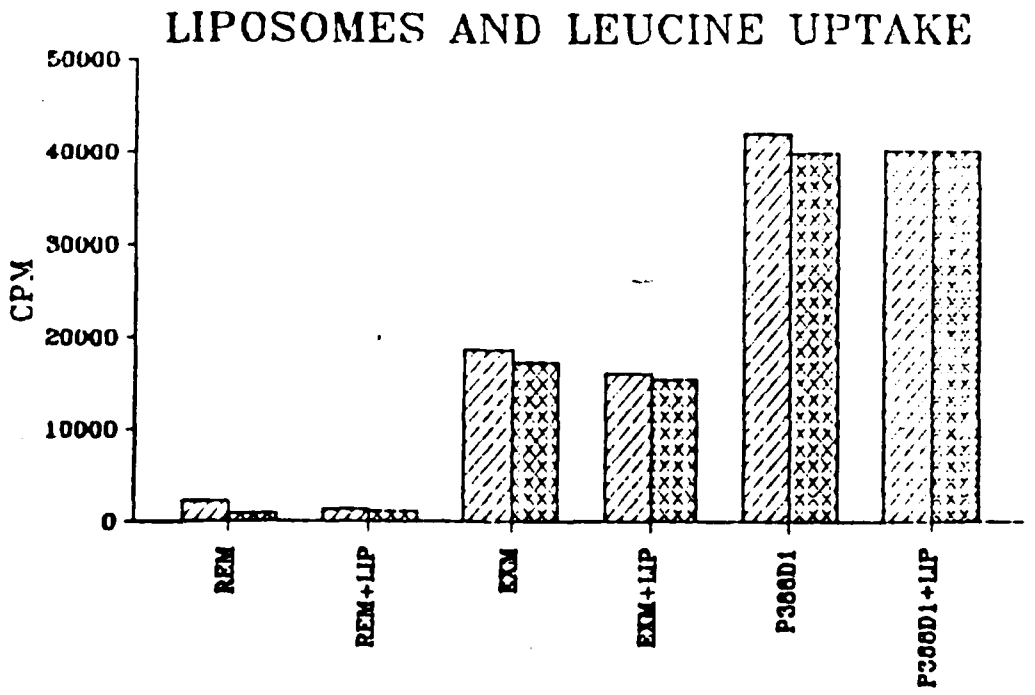
LIPOSOMES AND LEUCINE UPTAKE
CPM
50000
40000
30000
20000
10000
0
REM
REM+LIP
EXM
EXM+LIP
P388D1
P388D1+LIP
TYPE OF MACROPHAGE

Fig. 11.

2.0 Research Plan for the Next Project Period

2.1 Complete characterization of the immunomodulatory and host resistance enhancing activities of immunomodulators. We are initially evaluating each compound for host resistance enhancement against either EMC or HSV-2 infection, and against L. monocytogenes for some agents. For selected compounds we are also establishing their immunomodulatory activity for macrophages, as assessed by changes in ectoenzyme profiles, acquisition of antitumor activity, or changes in the macrophage-HSV interaction pattern.

2.2 Define the effects of immunomodulators on the prostaglandin-forming splenic MO in selectively depleted mice. We will complete the studies that have been performed using C. parvum in 89Sr and Sl/Sld mice. An approach to mechanism will be attempted through the use of interferons and selected lymphokines as well as bone marrow cells in efforts to define the mechanisms that regulate the capacity of splenic macrophages to synthesize and release prostaglandins. There is the obvious possibility that prostaglandin-forming macrophages normally inducible in the spleen are cells of bone marrow origin. Our approach will attempt to discriminate between an immigrant cell of bone marrow origin and a sessile bone marrow cell which elaborates a circulating regulatory agent.

2.3 Evaluate the toxic liposomes for depletion of MO. We plan to devote most of the effort toward the development of toxic liposomes. Methodological as well as experimental problems remain to be addressed. One approach is to develop methods of preparation and delivery of toxin-bearing liposomes that will result in enhanced toxicity. In theory, the introduction of the ricin A chain into a cell should be as lethal as the introduction of the whole ricin molecule, as reported by others (Dimitriadis, G.J. and Butters, T.D., FEBS LETTERS 98:33, 1979). A second pathway of development will be the increased use of freshly harvested mouse macrophages in vitro and the instillation of toxic liposomes intraperitoneally. In later experiments liposomes will be administered intravenously and their effect of hepatic and splenic macrophages monitored. The effects of additional toxic agents including astatine-211 will be evaluated.

In essence the goals for the forthcoming project period remain the same as outlined in the previous Progress Report. The difference is that we now have a greater number of experimental results which better define pitfals and areas in need of refinement. For example, we are currently in the process of trying to determine whether the apparently limited toxicity of our current preparation is a reflection of some degree of ric. A chain resistance inherent in P388D1 cells by the use of alternative targets. Experiments are also planned to see if liposome uptake can be enhanced by coupling IgG2A to the liposome vesicles in an attempt to promote endocytosis through the FcIgG2A receptor on macrophages. Although this procedure may reduce the selectivity of liposome uptake we currently see no pitfalls inherent in broadening the range of peritoneal cells destroyed by toxic inoculum. It appears that leucine uptake is not the most sensitive method of monitoring early cell damage in the target population currently under study. A variety of methods for the assessment of cell damage and death will be evaluated and compared.

An important aspect of this study that has not been addressed precisely in earlier proposals is the kinetics of recovery of a macrophage compartment following either partial or nearly total destruction. This is an area not only important in terms of macrophage origin and ontogeny but impacts also on microbicidal and tumoricidal function. The modulation of recovery by selected biologic response modifiers as set out in the original proposal still remains a goal of this project.

3.0 Publications/presentations related to this project.

3.1 Manuscripts

Shibata, Y. and A. Volkman. The effect of bone marrow depletion on prostaglandin E producing suppressor macrophages in mouse spleen. J. Immunol. 135:3897-3904, 1985.

Shibata, Y. and A. Volkman. The effect of hemopoietic microenvironment on suppressor macrophages in the congenitally anemic mice of the genotype Sl/Sld. J. Immunol. 135:3905-3910, 1985.

Shibata, Y., W.L. Dempsey, P.S. Morahan and A. Volkman. Selectively eliminated blood monocytes and splenic suppressor macrophages in mice depleted of bone marrow by strontium 89. J. Leuk. Biol. 38:659-669, 1985.

Morahan, P.S., A. Volkman, W.L. Dempsey, and J. Connor. Antimicrobial activity of various immunodulators: independence from normal levels of circulating monocytes and NK cells. Infec. Immun. 51:87-93, 1986.

Dempsey, W.L., A. Smith,and P.S. Morahan. Effect of inapparent murine hepatitis virus infections on macrophages and host resistance. J. Leuk. Biol. 39:559-566, 1986.

Morahan, P.S., E.R. Leake, D.J. Tenney, and M. Sit. Comparative analysis of modulators of non specific resistance against microbial infections. In: J. Majde, Ed. Immunological Adjuvants and Modulators of Non Specific Resistance to Microbial Infections (Alan R. Liss, NY).

Morahan, P.S., W.L. Dempsey, M.F. Sit, D.J.Tenney, E.R. Leake and J.R. Connor. Immune responses to virus infections: brief overview. Microbiologica, in press 1986.

Shibata, Y., A.P. Bautista, S.N. Pennington, J.L. Humes and A. Volkman. Eicosanoid production by peritoneal and splenic macrophages in mice depleted of bone marrow by 89Sr. Submitted to Am. J. Pathology.

3.2 Abstracts and presentations

Ackermann, M.F., W.L. Dempsey, P. Hwu, D. Tenney, E. Leake and P.S. Morahan. Effect of PMN depletion on MO activation by P. acnes. Eastern Pennsylvania Branch of the American Society of Microbiology, February 1985.

Tenney, D.J., M.F. Sit, E.R. Leake, M.T. Largen and P.S. Morahan. Effect of 89Sr on protein synthesis and intrinsic resistance of peritoneal macrohages to HSV-1. Reticuloendothelial Society annual meeting, August 1985.

Shibata, Y., A. Bautista, S. Pennington, J. Humes, and A. Volkman. Effect of bone marrow depletion with 89Sr on eicosanoid production by murine peritoneal and splenic macrophages. Int. Congress of Immunology, July 1986.

END

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